PROPERTIES OF HYDROGENASE FROM AZOTOBACTER VINELANDII[1]

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Since the discovery of hydrogenase in *Azotobacter vinelandii*, the enzyme has been studied in whole cells and in cell juices, chiefly with the Knallgas reaction and the reduction of methylene blue (Lee and Wilson, 1943; Lee, Wilson, and Wilson, 1942; Wilson, Lee, and Wilson, 1942; Wilson and Wilson, 1943). This paper reports a study of hydrogenase from *A. vinelandii* in cell-free extracts, its attempted purification, and the properties of the enzyme studied under simplified conditions. Similar studies have been made recently on the hydrogenases of other bacteria, particularly with *Rhodospirillum rubrum* and *Escherichia coli* (Gest, 1952; Joklik, 1950*a*, *b*).

METHODS

Preparation of extract. Azotobacter vinelandii, strain Original, was grown in Burk's nitrogen-free medium containing 2 per cent sucrose, to which nitrogen and oxygen were supplied by vigorous aeration. After 24 to 36 hours, the cultures were harvested and washed by centrifugation, and the cell pastes were frozen. Cell-free preparations were made by thawing and grinding 30 g of cell paste for 10 minutes with 25 g of alumina (Alcoa A303), extracting by diluting to 125 ml with 0.2 per cent potassium chloride, and centrifuging at 2,000 times gravity to remove the alumina and cellular debris. The cell-free extract thus obtained was recentrifuged at 29,000 times gravity, sedimenting a red-colored fraction containing subcellular particles with high specific activity. The cell-free particles were washed once in 0.2 per cent KCl by centrifugation, suspended in 0.005 M phosphate buffer, pH 6, and stored in stoppered test tubes in air at −20 C. All manipulations including centrifugation were made at 0 to 2 C. From 30 g of cell paste ground with alumina, the usual yield was 40 ml of resuspended washed particles, containing 4,000 units (see next section) with specific activity 25,000 (microliters H_2 transferred to acceptor)/(hr × mg protein nitrogen). The most active preparation had a specific activity of 36,000. One-tenth ml of the suspensions sufficed for a single manometer vessel.

Hydrogen acceptors. Washed cell-free particles from *A. vinelandii* catalyze the reduction of a variety of hydrogen acceptors over a wide range of redox potential. The following compounds are among those which accept hydrogen in the presence of azotobacter hydrogenase: benzyl viologen (E_0' at pH 7 and 30C = − 0.359 v); methylene blue (E'_0 = +0.011 v); 2,6-dichlorophenolindophenol (E'_0 = +0.217 v); cytochrome C (E'_0 = +0.262 v); ferricyanide (E'_0 = +0.360 v); and oxygen (E'_0 = +0.815 v). The rates of reduction vary, as would be expected; the most rapid uptake of hydrogen (measured manometrically) is with ferricyanide and methylene blue. Several compounds did not serve as hydrogen acceptors in the presence of washed cell-free particles, including nitrate, fumarate, pyruvate, α-ketoglutarate, diphosphopyridine nucleotide, and riboflavin.

The optimum pH for uptake of hydrogen with ferricyanide as electron acceptor was pH 8. This optimum was obtained with phosphate, glycylglycine, diethylbarbiturate, and borate buffers, under conditions where the enzyme was not exposed to the buffer until the assay was begun, to avoid any effect of pH on enzyme stability. The activities in the various buffers at pH 8 were equal, and the addition of phosphate had no effect on the activity in other buffers.

The concentration of ferricyanide necessary to give half-maximal velocity was 0.003 M (phosphate buffer, pH 8, at 33 C); this was determined from the Lineweaver-Burk plot of the reciprocal of the velocity against the reciprocal of the sub-

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strate concentration. A final concentration of 0.05 M ferricyanide was adopted for routine manometric assay of hydrogenase. The hydrogen pressure resulting in half-maximal velocity was approximately 0.05 atmosphere.

Method of assay. The conditions for routine assay of hydrogenase were:

Main compartment:	
Potassium phosphate buffer, pH 8, 0.2 M	1.5 ml
Potassium ferricyanide, 0.3 M	0.5 ml
Distilled water	0.5 ml
Side arm:	
Enzyme; water to make	0.5 ml
Center well:	
Potassium hydroxide, 20 per cent	0.2 ml
H_2 atmosphere[3]; 33 C	

Specific activity was expressed in terms of $Q_{H_2}(N)$ or (microliters H_2 taken up)/(mg nitrogen × hours). When ferricyanide was used as the hydrogen (electron) acceptor, the term $Q_{FeCy}(N)$ is used to designate (microliters hydrogen transferred to ferricyanide)/(mg nitrogen × hr). One unit was defined as the amount of enzyme necessary to catalyze the uptake of 100 microliters H_2 per hour.

The rate of reaction was found to be independent of the rate of diffusion of hydrogen to the enzyme, under the conditions selected for routine assay. Hydrogen uptake was directly proportional to enzyme concentration over a wide range. Prolonged incubation of the enzyme under hydrogen before assay had no effect on the activity found.

RESULTS

Purification. Separation of particles. The first obstacle to purification of hydrogenase in extracts of *A. vinelandii* is the association of the enzyme with cellular particles (table 1). Centrifugation of a suspension of alumina-ground cells at 2,000 times gravity results in precipitation of 70 to 80 per cent of the total activity in a fraction containing cells and cellular debris. The cell-free extract (supernatant from 2,000 G) contains about 20 per cent of the total activity, but most of that too is associated with small particles that can be sedimented at 29,000 G. A small proportion of the hydrogenase remains in the supernatant after 30 minutes at 76,000 times gravity. In different experiments, this soluble fraction ranges from 4 to 10 per cent of the total activity in macerated cells, or from 20 to 25 per cent of the activity in cell-free extracts.

No attempt was made to purify the soluble (nonsedimentable) fraction of the hydrogenase because of its low specific activity and yield. Instead, attempts were directed toward solubilizing the much larger fraction bound to cell particles. Several techniques for solubilizing and purifying the hydrogenase in particles of *A. vinelandii* were attempted, without success. The enzyme in cell-free particles was stable to treatment with weak acids and bases, repeated freezing and thawing, salt fractionation, and fractionation with ethyl and methyl alcohol, but remained particle-bound and insoluble. The enzyme was inactivated (based on assay with ferricyanide) by exposure to toluene, ethyl acetate, butyl alcohol, sodium choleate, and by drying with acetone or by lyophilization.

With all attempts at removing hydrogenase from cell particles having proved unsuccessful (as judged by the H_2-ferricyanide assay), attention was turned to the properties of the enzyme as it exists in the bound form on the particles. This study has the disadvantage that the conditions are complex and probably involve more than one enzyme, but has the virtue that the results probably have greater significance for the reactions that occur physiologically (*cf*, study of mitochondrial preparations from animal tissue).

Appearance. The enzyme used for these studies was obtained from alumina-ground cells. The fraction taken was that not sedimented at 2,000 times gravity but sedimented at 29,000 times gravity. This fraction contains 10 per cent of the total activity of macerated cells and has a specific activity of 22,000 to 28,000 (microliters H_2 transferred to ferricyanide in an hour per mg nitrogen) at 33 C. When packed by centrifugation, the particles are red, and suspensions have a pinkish tinge. Examination with the light microscope revealed nothing of characteristic size or shape. Smears are gram negative. Individual particles are too small to distinguish, but after freezing they tend to clump together into visible granules.

Stability. The hydrogenase of washed cell-free

[3] Gassing with hydrogen was accomplished by evacuating manometers to 0.1 atmosphere, filling to atmospheric pressure with H_2 (stored over alkaline pyrogallol), and repeating the evacuation and filling twice.

particles from *A. vinelandii* is more stable than hydrogenase preparations of other bacteria (*R. rubrum*, *E. coli*) that have been described, which are rapidly inactivated by air. In one trial lasting 18 days, the enzyme suffered no significant loss of activity, despite the continuous exposure to air; the only precaution was to maintain the enzyme at 0 C when thawed, or at −20 C when stored overnight. The assay varied from 10,200 to 11,800 μL H_2 absorbed by a ml of enzyme preparation per hr with an average of 10,800 ± 400 μL. The enzyme in washed particles does lose activity gradually over a period of several weeks to months. In crude cell-free juices, hydrogenase becomes inactive within one to two weeks. A suspension of particles in 0.1 M phosphate buffer at pH 6 retained 70 per cent of its activity after heating at 60 C for 3 minutes.

Chemical properties. Coenzymes. The common cofactors involved in hydrogen transfer and other enzyme reactions do not appear to be required in the hydrogenase particle-ferricyanide system. The following additions to preparations of washed particles had no effect on the rate of hydrogen uptake with ferricyanide: diphosphopyridine nucleotide; triphosphopyridine nucleotide; flavin adenine dinucleotide; riboflavin; adenosine triphosphate; adenylic acid; folic acid; boiled extract (Kochsaft) of *A. vinelandii* cells; magnesium ion; orthophosphate; methylene blue; cytochrome C; and muscle cytochrome oxidase.

Additional evidence contraindicating any close connection between hydrogenase and the co-enzymes of hydrogen transfer comes from spectrophotometric studies. Experiments were designed to determine whether or not hydrogenase, with hydrogen, can reduce diphosphopyridine nucleotide, triphosphopyridine nucleotide, flavin adenine dinucleotide, or riboflavin. The possible reduction of added diphosphopyridine nucleotide and triphosphopyridine nucleotide was measured by the increase in absorption of light at 340 millimicrons, and reduction of added flavin adenine dinucleotide or riboflavin was measured by the decrease in absorption at 450 millimicrons. Hydrogenase and coenzyme were suspended in cuvettes fitted with plugs, and hydrogen was bubbled into the suspensions through a capillary. After gassing one minute, the cuvettes were stoppered and the absorption was followed at the appropriate wavelength. As a control, hydrogenase and a small amount of ferricyanide were gassed, and the reduction of ferricyanide was followed at 420 millimicrons. The ferricyanide was reduced, but under identical conditions, no detectable change occurred in absorption with diphosphopyridine nucleotide, triphosphopyridine nucleotide, flavin adenine dinucleotide, or riboflavin. Variation of the pH between pH 7 and pH 8 and the addition of cyanide and arsenate to the hydrogenase-diphosphopyridine nucleotide system did not cause reduction of diphosphopyridine nucleotide. Similar results with bacterial hydrogenase have been reported by Gest (1952).

Although one or more of these cofactors may be required for the H_2-ferricyanide reaction, addi-

TABLE 1

Distribution of hydrogenase activity in extracts of Azotobacter vinelandii

GRAVITATIONAL FORCE	DISTRIBUTION OF ACTIVITY AFTER CENTRIFUGATION			
	Precipitate		Supernate	
	Units	$Q_{FeCy}(N)$	Units	$Q_{FeCy}(N)$
Alumina-ground cells at 900 G, 15 min	0 (Alumina)	0	100	10,700
Supernate of above at 2,000 G, 20 min	70 (Cell debris)	14,800	22 (Cell-free extract)	5,400
Supernate of above at 16,500 G, 20 min	4 (Particles)	19,800	16	4,080
Supernate of above at 29,000 G, 20 min	4 (Particles)	26,000	5	1,500

tion of these is unnecessary because of the type of integration in the particles analogous to that in the mitochondria of animal tissues. The present experimental data, however, offer no direct evidence for postulating that any of the common known coenzymes are linked closely to hydrogenase.

Reduction of cytochrome C. Although cytochrome C has no effect when added to the H_2-ferricyanide assay system, it is reduced in the presence of hydrogenase, a fact which may be significant for the metabolism of hydrogen in *A. vinelandii*. Figure 1A shows the reduction of cytochrome C, as followed by measuring the

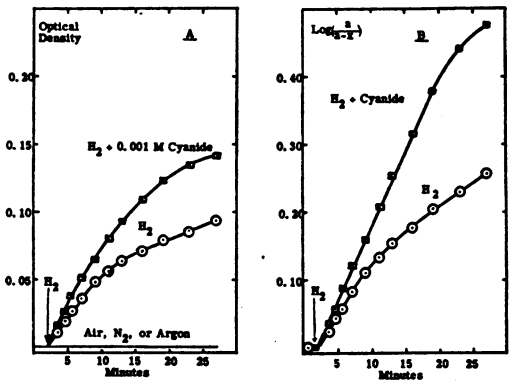

Figure 1. Reduction of cytochrome C by hydrogenase. 1A. Reduction of cytochrome C by washed particles of azotobacter in pH 7.45 phosphate buffer measured by increase in absorption at 550 millimicrons; cytochrome C concentration 1.1×10^{-5} M. 1B. Kinetics of the reduction of cytochrome C; a = initial concentration of cytochrome C, and x = amount of cytochrome C reacting in time t.

increase in optical absorption at 550 millimicrons. The system contained washed particles bearing hydrogenase, suspended in phosphate buffer at pH 7.45, with cytochrome C at 1.1×10^{-5} M and cyanide (when present at 1×10^{-3} M); the atmosphere was air, hydrogen, nitrogen, or argon. Reduction occurred in the presence of hydrogen (with or without cyanide), but not in the others. Cytochrome C is not reduced by hydrogen in the absence of hydrogenase, nor with boiled suspensions of hydrogenase. The effect of cyanide on accelerating the reduction of cytochrome C is possibly a consequence of inhibition of cytochrome oxidase, which in the presence of traces of oxygen would oxidize reduced cytochrome C.

Analysis of the kinetics of the reduction of cytochrome C indicates that the reaction initially is first order with respect to oxidized cytochrome C (figure 1B). Calculated from the initial straight portion of the curves in figure 1B, the first order velocity constant, k, for the reduction of cytochrome C by hydrogenase is 0.039 min^{-1} without cyanide, and 0.051 min^{-1} with cyanide. The first order velocity constants for the reduction of cytochrome C and of ferricyanide are of the same order of magnitude. In an experiment in which

TABLE 2

Cyanide inhibition of hydrogenase

EXPERIMENT	CYANIDE CONCENTRATION			
	0.001 M	0.005 M	0.01 M	0.04 M
	Per cent inhibition			
1 Not pre-incubated*	6	—	—	—
2 Not pre-incubated*	—	—	53	67
3 Not pre-incubated*	0	11	10	—
4 Pre-incubated†	90	99	100	—
5 Pre-incubated†	—	—	99	—
6 Pre-incubated†	—	—	90	—

* Not pre-incubated:
Main chamber: Phosphate, pH 8, 300 micromoles; cyanide as indicated; ferricyanide, 150 micromoles; water to 2.5 ml.
Side arm: Enzyme, water to 0.5 ml.
† Pre-incubated:
Main chamber: Phosphate, pH 8, 300 micromoles; enzyme; cyanide as indicated; water to 2.5 ml.
Side arm: Ferricyanide, 150 micromoles (0.5 ml).
Both:
Center well: H_2O, 0.2 ml. 33 C; hydrogen atmosphere.

the reduction of 3.3×10^{-4} M ferricyanide was followed spectrophotometrically at 420 millimicrons, under conditions otherwise comparable to those used for the reduction of cytochrome C, the first order velocity constant, k, was 0.177 min^{-1}, as compared to k of 0.039 min^{-1} for cytochrome C at 1.1×10^{-5} M.

Effect of inhibitors. Carbon monoxide inhibited the rate of the H_2-ferricyanide reaction approximately 45 per cent when H_2/CO was 4, and by approximately 65 per cent at $H_2/CO = \frac{1}{3}$. The inhibition was noncompetitive and was not affected by the light from a pair of reflector lamps

(total 1,000 watts) within 14 cm of the bottoms of the manometer flasks. Under similar conditions this illumination reversed completely the carbon monoxide-inhibition of yeast respiration. The inhibition of azotobacter hydrogenase by carbon monoxide evidently differs from the inhibition of yeast cytochrome oxidase, and, accordingly, the inhibited component in the H_2-ferricyanide reaction is not wholly analogous to the iron porphyrin of cytochrome oxidase.

Cyanide inhibits azotobacter hydrogenase more when the exposure is made in air than under hydrogen (table 2). When the enzyme was kept from the cyanide in the manometer vessel until to having run the experiment at pH 8. These concentrations of azide overlap the range in which Wilson and Wilson (1943), studying the Knallgas reaction, found both stimulation (at lower levels) and inhibition (higher levels).

Hydroxylamine is oxidized by ferricyanide and cannot be used as an inhibitor of the H_2-ferricyanide reaction.

Phosphate esterification in the Knallgas reaction. The Knallgas reaction ($H_2 + \frac{1}{2}O_2 = H_2O$) in crude cell-free extracts of *A. vinelandii* leads to the disappearance of orthophosphate in the presence of adenylic-5-phosphate, and the missing phosphate is recoverable upon boiling 7 minutes

TABLE 3

Phosphate esterification in the Knallgas reaction

	PHOSPHATE CONCENTRATION, MICROMOLES PER CUP						
	Zero time			After 55' Knallgas reaction			
	Ortho-P* (1)	Ortho-P + Δ7'P (2)	Δ7'P (3)	Ortho-P* (4)	Ortho-P + Δ7'P (5)	Δ7'P (6)	Ortho-P (1–4)
Control	21.8	22.2	0.4	16.6	23.1	6.5	5.2†
plus 9.2 μM diphosphopyridine nucleotide	22.2	—	—	17.6	—	—	4.6
plus 30 μM NaF	23.0	22.9	0.0	17.6	22.9	5.3	5.4

* The Lowry-Lopez and the Fiske-Subbarow methods for ortho-phosphate gave similar values. All subsequent analyses were by the simpler method of Fiske-Subbarow.

† P/O ratio = 0.24.

Main chamber: Phosphate, pH 7.1, 20 micromoles; $MgCl_2$, 10 micromoles; adenylic-5-phosphate (Bischoff), 16 micromoles; diphosphopyridine nucleotide, fluoride, as indicated; water to 2.5 ml.

Side arm: Cell-free juice of *Azotobacter vinelandii*, 0.5 ml (1.5 mg N).

Center well: H_2O, 0.2 ml.

38 C; atmosphere 80 per cent H_2, 20 per cent air.

all air had been replaced by hydrogen, cyanide at 0.001 M inhibited 0 to 6 per cent, and at 0.01 M inhibited 53 per cent. When the enzyme and cyanide were mixed together in the presence of air ("pre-incubated", table 3), cyanide at 0.001 M inhibited 90 per cent, and at 0.01 M inhibited up to 100 per cent. The enhancement of cyanide inhibition (and of other inhibitors) by air has been noted by several workers with hydrogenase from other sources, and has been interpreted to mean that only the oxidized form of hydrogenase combines with iron complexing inhibitors.

Azide, if anything, stimulated slightly the H_2-ferricyanide reaction at concentrations from 0.005 to 0.06 M, with or without pre-incubation in air. Since azide is known to be a poor inhibitor at high pH, the lack of inhibition may be referable in normal hydrochloric acid (tables 3 and 4). After a 55 min period of Knallgas activity (table 3), during which 65 microatoms of combined H_2 and O_2 were taken up, the orthophosphate decreased from approximately 22 to 17 micromoles, and this decrease was balanced by the formation of an equivalent quantity of acid-hydrolyzable phosphate. Comparable results were obtained in another experiment (not shown), in which additions of glucose, pyruvate, and glycerol were ineffective in increasing the uptake of phosphate.

The necessity for adenylic-5-phosphate is shown in table 4. Some phosphate was taken up in the absence of added acceptor, indicating that an endogenous acceptor is available. Yeast adenylic acid (adenylic-3-phosphate) was unsuit-

able as an acceptor, but muscle adenylic acid (adenylic-5-phosphate) definitely enhanced the disappearance of orthophosphate. Phosphate esterification has not been reported previously for the Knallgas reaction in cell-free preparations.

The H_2-ferricyanide reaction was not accompanied by detectable uptake of phosphate under conditions for routine assay (modified by additions of magnesium ion, adenylic acid, hexokinase, and glucose), possibly because the centrifugation and washing of the cell-free particles removed essential accessory enzymes. The occurrence of phosphate esterification in the H_2-ferricyanide reaction with crude juices was not investigated.

TABLE 4

Effect of adenylic acid on phosphate esterification in the Knallgas reaction

	ZERO-TIME ORTHO-P	AFTER 60′ ORTHO-P	Δ ORTHO-P
	μM	μM	μM
Control	21.5	20.4	1.1
plus 4 μM Ad-3-P	21.5	21.0	0.5
plus 4 μM Ad-5-P	21.7	17.8	3.9*

* P/O = 0.25.

Main chamber: Phosphate, pH 7.1, 20 micromoles; $MgCl_2$, 10 micromoles; glucose, 20 micromoles; hexokinase, 50 micrograms; adenylic acids as indicated; water to 2.5 ml.

Side arm: Cell-free juice, 0.5 ml (1.23 mg N).

Center well: H_2O, 0.2 ml.

38 C; atmosphere 80 per cent H_2, 20 per cent air.

Physical properties. Absorption spectrum. The absorption spectrum of a suspension of hydrogenase bearing particles gives a possible clue concerning the action of the enzyme (figure 2). The upper curve in figure 2 is for a suspension freshly prepared by fractional centrifugation, resuspended in pH 6 phosphate buffer and observed in air. Absorption maxima are found in the regions of 400, 410, and 550 mμ. The lower curves (figure 2) are for the same suspension, reduced with thiosulfate and oxidized with ferricyanide, the absorptions having been corrected for that of thiosulfate and ferricyanide, respectively. A sharp peak at or near 410 mμ now is seen plainly with both oxidized and reduced enzyme. A peak in this vicinity (the Soret band) has been described in some hemoproteins. The absorption in the region, 500 to 580 mμ, is broad, reaching a maximum at 555 mμ, and is a property only of the reduced form of the particles. Absorption in this region is characteristic of hemoproteins and cytochromes, and the absorption at 555 mμ is close to the absorption maximum of cytochrome C. The cytochrome material accounts for the red color of the particles. For convenience in description, the material absorbing at 555 mμ will be referred to as a "cytochrome component" and the material

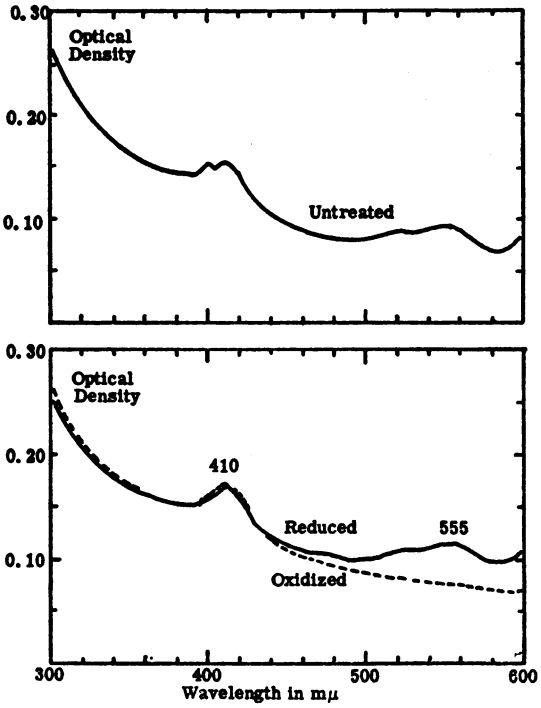

Figure 2. Absorption spectra of oxidized and reduced cell-free particles from *Azotobacter vinelandii*. Upper figure, absorption by a suspension of fresh washed particles of azotobacter in pH 6 phosphate buffer; observed in air. Lower figure, the same suspensions reduced with thiosulfate and oxidized with ferricyanide; absorptions corrected for absorption by thiosulfate or ferricyanide.

absorbing at 410 mμ will be referred to as a "hemoprotein", although there is no direct evidence that either component is correctly named.

The hemoprotein component absorbs maximally at 410 mμ under oxidizing conditions, and at 415 mμ under reducing conditions. The shift in absorption from 410 to 415 mμ can be demonstrated by passing hydrogen through a suspension oxidized by air or by ferricyanide. The hemoprotein component evidently exists in an oxidized

and a reduced form, and the reduction is accomplished by hydrogen. Possibly, this component actually represents hydrogenase, but we have no direct evidence of its identity; from the available evidence the hemoprotein equally well could be a carrier linked to hydrogenase, or even a component entirely independent of hydrogenase which is reduced nonspecifically, as is methylene blue or ferricyanide. No absorption maximum was observed in the reduced spectrum at 340 mμ (pyridine nucleotide coenzymes), nor did the oxidized spectrum have a maximum at 450 mμ (flavoprotein components).

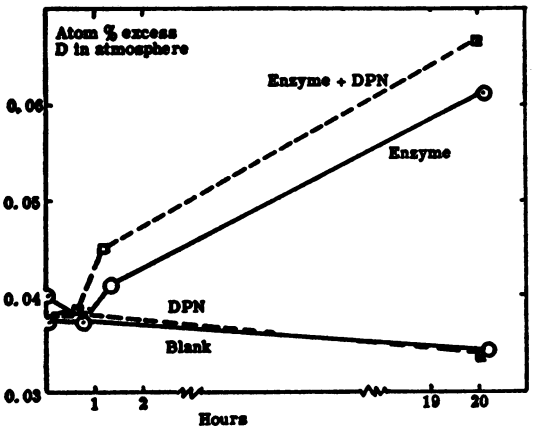

Figure 3. The exchange reaction in cell-free preparations from azotobacter. Contents of Thunberg tubes: Main tube contains phosphate buffer, pH 7.5, 100 micromoles; 1.0 ml D_2O, 38 atom per cent excess D; 0.3 micromoles diphosphopyridine nucleotide (DPN) when used; water to 2.0 ml. Cap of tube contains 1.0 ml enzyme (washed cell-free particles containing 0.2 mg N), Q_{FeCy} (N) = 26,500. Temperature 30 C. Atmosphere H_2. Final concentration of D in D_2O after mixing, 13 atom per cent excess.

Exchange with deuterium. Washed particles from azotobacter catalyze a slow exchange between gaseous hydrogen and deuterium water, but the exchange is much slower than that reported for whole cells of *Proteus vulgaris* (Hoberman and Rittenberg, 1943; Farkas and Fischer, 1947), *E. coli* (Farkas, 1936), and *R. rubrum* (Johnston and Frenkel, 1951).

Results of an experiment showing the rate of appearance of deuterium in the atmosphere from D_2O are given in figure 3. Thunberg tubes were prepared containing D_2O in the main part of the tube and enzyme in the cap. Other tubes contained additions of diphosphopyridine nucleotide or benzyl viologen. Duplicate tubes without enzyme served as controls. The tubes were evacuated, filled with H_2, re-evacuated and refilled with H_2, and were incubated at 30 C without shaking. The content of deuterium appearing in the atmosphere was determined at intervals with a Consolidated-Nier mass spectrometer. As seen in figure 3, the rate of exchange was very low, despite the use of a quantity of enzyme ten times that adequate for rapid uptake of hydrogen in manometric experiments. Addition of diphosphopyridine nucleotide to the enzyme apparently increased the rate of exchange, but the rates were so low that the effect probably is not significant. Possibly the rate of exchange could be increased by modification of the experimental conditions, such as by more complete removal of oxygen and by shaking the gas over a thin layer of deuterium water.

Miscellaneous. The *temperature characteristic* (μ) of the H_2-ferricyanide reaction is 15,500 calories, calculated from the rates of reaction at three temperatures: 22 C, 30 C, and 37 C. This temperature characteristic corresponds to a Q_{10} of 2.4 in the region 25 C to 35 C. The temperature characteristic for the H_2-ferricyanide reaction is higher than that found for the Knallgas reaction in azotobacter, 11,200 calories, (Wilson, Lee, and Wilson, 1942), but it is very close to the figure (15,200 calories) found for the exchange reaction and for the H_2-fumarate reaction, in cells of *P. vulgaris*, by Farkas and Fischer (1947).

The *turnover number* of an enzyme is the number of molecules of substrate decomposed per molecule of enzyme in one minute. Assuming a molecular weight of 100,000 for hydrogenase, and a purity of one per cent in the washed particles, the turnover number is 30,000 for a preparation of specific activity 25,000.

The *electromotive force* of the over-all reaction

$$\tfrac{1}{2}H_2 + Fe(CN)_6^{\equiv} \rightarrow Fe(CN)_6^{\equiv} + H^+$$

under the conditions of assay (pH 8, 33 C, one atmosphere of H_2), and at equal concentrations of ferricyanide and ferrocyanide, was calculated to be 0.85 volts. This emf corresponds to a free energy change

$$\Delta F = -n\,F\,E = -19{,}600 \text{ calories/mole},$$

and the equilibrium constant, calculated from the equation

$$\Delta F = -RT \ln K$$

was

$$K = 10^{14}.$$

DISCUSSION

The hydrogenase catalyzed exchange between hydrogen and deuterium water is perhaps the most nearly ideal system for the study of hydrogenase (Hoberman and Rittenberg, 1943; Farkas and Fischer, 1947), but the reaction has not been studied commonly because of a lack of the necessary facilities. Exchange by cell-free particles from *A. vinelandii* was very low (figure 3) under the conditions used. Most experiments on hydrogenase have been with hydrogen acceptors, such as methylene blue or other dyes, ferricyanide, nitrate, fumarate, or oxygen. Interpretation of results from these studies is handicapped by the necessity for carriers or enzymes to activate the acceptors. Some observations that have been reported may be characteristic of the acceptor-activating enzyme rather than of hydrogenase.

The hypothesis that hydrogenase is an iron porphyrin enzyme stems from a study of the exchange reaction in suspensions of *P. vulgaris* by Hoberman and Rittenberg (1943), who made the following observations: (a) the enzyme was inhibited by carbon monoxide and the inhibition was reversed partially by light (resembling the iron porphyrin of cytochrome oxidase); (b) the enzyme was inactivated in air but was restored to activity by hydrogen or by incubation with substrates (indicating a reversibly oxidizable component necessary for activity); (c) the enzyme was inhibited by 0.001 M cyanide when the cyanide was added in the presence of air (followed by hydrogen) but was not inhibited by 10 times that amount of cyanide when it was added to the active, exchanging system under hydrogen, suggesting that the oxidizable component was iron, which combined with cyanide when in the ferric state but not in the ferrous state, and that its action did not involve an oscillation between the ferric and ferrous states.

The inhibition of hydrogenase by carbon monoxide has been confirmed repeatedly with both whole cells and purified cell-free extracts, but reversal of the inhibition by light has not been duplicated, either in the exchange reaction (Farkas and Fischer, 1947), or in the reduction of methylene blue (Lascelles and Still, 1946; Joklik, 1950*b*), ferricyanide, or oxygen (Wilson and Wilson, 1943). The evidence for an iron porphyrin structure wholly analogous to that of cytochrome oxidase must be considered incomplete.

The inactivation of hydrogenase by exposure to air and preservation or restoration of activity by hydrogen, reducing agents, or substrates also have been noted repeatedly (Back, Lascelles, and Still, 1946; Farkas and Fischer, 1947; Joklik, 1950*a*; Gest, 1952), but the hydrogenase in washed cell-free particles of *A. vinelandii* is much more stable. This confirmation is consistent with the hypothesis that the reversibly oxidizable component is iron, but does not exclude another possibility: that it may be an exposed sulfhydryl group, as suggested by Joklik (1950*a*).

The third observation, that cyanide inhibits more strongly when added to enzyme exposed to air (followed by hydrogen) than when added to enzyme already under hydrogen, also has been confirmed several times (table 2; Lascelles and Still, 1946; Farkas and Fischer, 1947), and a similar type of inhibition has been noted with hydroxylamine, fluoride, and other inhibitors. Joklik (1950*b*), however, reports exactly opposite results with cyanide. If the effect of cyanide is interpreted to mean that an iron component must be reduced for hydrogenase to be active, and that its function does not involve a ferrous-ferric valence change, one might expect that an inhibition of the reduced enzyme could be demonstrated with substances able to complex with ferrous iron. This has not been possible with ortho-phenanthroline, α-α'-dipyridyl, or dithizone (Lascelles and Still, 1947; Gest, 1952).

Several other observations have a possible significance for the question of iron or iron porphyrins being part of hydrogenase. Waring and Werkman (1944) reported that *Aerobacter indologenes* grown in an iron-deficient medium had low hydrogenase activity (as well as formic dehydrogenase and formic hydrogenlyase), in comparison to cells grown in an identical medium with more iron. Hydrogenase is linked enzymatically with iron porphyrin enzymes, as shown by the fact that nitrate and oxygen serve as hydrogen-acceptors, both of which require iron porphyrin enzymes for activation. The reduction of cytochrome C by cell-free particles from *A. vinelandii* (figure 1) is another example of a link

between hydrogenase and iron porphyrins. A third example is seen in the absorption spectrum of particles from *A. vinelandii* (figure 2), where a hemoprotein component and a cytochrome component are found to be reduced in the presence of hydrogen.

The foregoing observations lead to the conclusion that hydrogenase, if not an iron porphyrin enzyme itself, at least functions in conjunction with such enzymes and carriers. This evidence, together with the previously demonstrated inhibition of the exchange reaction (which should not require the mediation of other enzymes) by cyanide and carbon monoxide (Hoberman and Rittenberg, 1943; Farkas and Fischer, 1947), as well as the inability to demonstrate any close relationship to the common coenzymes of metabolic hydrogen transfer, makes it appear more likely that hydrogenase is an iron enzyme. Evidence for a porphyrin structure is less certain. Admittedly, most of these observations constitute only indirect evidence so that the conclusion can be only tentative until more highly purified preparations of hydrogenase becomes available.

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SUMMARY

Hydrogenase in cell-free juices of *Azotobacter vinelandii* was found to be associated largely with small particles. With ferricyanide as hydrogen-acceptor, maximal activity was shown at pH 8, and half-maximal activity at 0.003 M ferricyanide and at 0.05 atmosphere of hydrogen.

Hydrogenase activity was stable in such centrifuged, washed cell-free particles, and was not affected by additions of diphosphopyridine nucleotide, triphosphopyridine nucleotide, flavin adenine dinucleotide, riboflavin, or Kochsaft of whole cells; and none of the coenzymes was reduced by hydrogenase. Cytochrome C was reduced by the hydrogenase of washed particles, and the absorption spectrum of the particles revealed a component corresponding to cytochrome C. The absorption spectrum also indicated the presence of a hemoprotein component having a peak at 410 millimicrons, which shifts to 415 millimicrons in the presence of hydrogen. The hydrogenase of washed particles was inhibited by carbon monoxide, but the inhibition was not affected by light (1,000 watts). Cyanide inhibited more strongly when added in air than in hydrogen.

The exchange reaction was very slow. The temperature characteristic, turnover number, and the equilibrium constant based on the standard free energy for the hydrogen-ferricyanide reaction were estimated. Crude juices carried out the Knallgas reaction with esterification of phosphate via adenosine-5-phosphoric acid.

The results were discussed in terms of the hypothesis that hydrogenase is an iron porphyrin enzyme.

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